Localization and role of NPC1L1 in cholesterol absorption in human intestine

Alain Théophile Sané,* Daniel Sinnett,† Edgard Delvin,§ Moïse Bendayan,** Valérie Marcil,* Daniel Menard,†† Jean-François Beaulieu,†† and Emile Levy1,*

Departments of Nutrition,* Pediatrics,† Biochemistry,§ and Pathology and Cell Biology,** CHU-Sainte-Justine, and University of Montreal, Montreal, Quebec H3T 1C5, Canada; and Group on the Functional Development and Physiopathology of the Digestive Tract,†† Canadian Institute of Health Research, and Department of Cellular Biology, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, Quebec J1H 5N4, Canada

Abstract Recent studies have documented the presence of Niemann-Pick C1-Like 1 (NPC1L1) in the small intestine and its capacity to transport cholesterol in mice and rats. The current investigation was undertaken to explore the localization and function of NPC1L1 in human enterocytes. Cell fractionation experiments revealed an NPC1L1 association with apical membrane of the enterocyte in human jejunum. Signal was also detected in lysosomes, endosomes, and mitochondria. Confirmation of cellular NPC1L1 distribution was obtained by immunocytochemistry. Knockdown of NPC1L1 caused a decline in the ability of Caco-2 cells to capture micellar [14C]free cholesterol. Furthermore, this NPC1L1 suppression resulted in increased and decreased mRNA levels and activity of HMG-CoA reductase, the rate-limiting step in cholesterol synthesis, and of ACAT, the key enzyme in cholesterol esterification, respectively. An increase was also noted in the transcriptional factor sterol-regulatory element binding protein that modulates cholesterol homeostasis. Efforts were devoted to define the impact of NPC1L1 knockdown on other mediators of cholesterol uptake. RT-PCR evidence is presented to show the significant decrease in the levels of ABCA1, ABCG5, and cluster determinant 36 in NPC1L1-deficient Caco-2 cells. Together, our data suggest that NPC1L1 contributes to intestinal cholesterol homeostasis and possibly cooperates with SR-BI to mediate cholesterol absorption in humans.—Sané, A. T., D. Sinnett, E. Delvin, M. Bendayan, V. Marcil, D. Ménard, J-F. Beaulieu, and E. Levy. Localization and role of NPC1L1 in cholesterol absorption in human intestine. J. Lipid Res. 2006. 47: 2112–2120.

Supplementary key words enterocyte • 3-hydroxy-3-methylglutaryl-coenzyme A reductase • acyl-coenzyme A:cholesterol acyltransferase • scavenger receptor class B type I • sterol-regulatory element binding protein • cluster determinant 36 • ATP-binding cassette transporter A1 • ATP-binding cassette transporter G5 • Niemann-Pick C1-Like 1

Coronary heart disease is the most important clinical manifestation of atherosclerosis and remains the main cause of death in developed societies. Hypercholesterolemia is beyond doubt the most prominent risk factor for the development of atherosclerosis, which is caused by derangements in cholesterol homeostasis (i.e., intestinal uptake, endogenous synthesis and metabolism, transport in lipoprotein particles, and biliary excretion). At present, there are increasing efforts to understand the physiology of intestinal fat transport in view of the positive relationship between cholesterol absorption, plasma cholesterol levels, and coronary heart disease (1–4). Additionally, augmented dietary fat and cholesterol intake have been tightly linked to the increased incidence of other diseases, such as cancer, diabetes, and obesity (5–7).

In stark contrast to previous tenets suggesting that cholesterol uptake occurs as a passive diffusion down a concentration gradient, more and more investigators now support protein-mediated cholesterol absorption. Curiously, protein transporters intervening in intestinal cholesterol movement have not yet been ascertained. A few recent studies have proposed scavenger receptor class B type I (SR-BI) as a candidate protein involved in dietary cholesterol transport (8–11). On the other hand, the involvement of SR-BI in cholesterol uptake has been questioned because intestinal cholesterol absorption was shown to be unaffected by deletion of the SR-BI gene in mice (10, 12). Other laboratories suggested that cluster determinant 36 (CD36) may provide a conduit for the movement of cholesterol from the brush-border membrane into the intestinal mucosa (13), but such assumptions have not been confirmed.
by in vitro and in vivo studies. Using a genetic approach, Niemann-Pick C1-Like 1 (NPC1L1) has been identified as a critical mediator of cholesterol absorption (14). Indeed, NPC1L1 has been found abundantly expressed in animal intestine, and mice deficient in NPC1L1 displayed marked reductions in sterol absorption (14). Furthermore, genetic variation in NPC1L1 appeared to contribute to variability in cholesterol uptake and plasma levels of low density lipoproteins (15). However, there were only limited biochemical and immunocytochemical analyses revealing NPC1L1 in the gut. To our knowledge, studies have mostly been carried out in animals, and more valuable information pertaining to intestinal tissues is required to appreciate the role of NPC1L1 in gut sterol homeostasis in humans.

To gain insight into the localization of NPC1L1 in human intestine, an immunoelectron microscopy approach was undertaken using protein A-gold coupled to specific antibodies. To lend further support to immunocytochemistry, subcellular fractionation was carried out to examine NPC1L1 protein expression. Molecular strategies were also devised in the Caco-2 cell line, a reliable human intestinal model for lipid metabolism and lipoprotein assembly (16, 17), to determine the importance of NPC1L1 in intestinal cholesterol transport and metabolism. Finally, studies were performed to establish the relationship between NPC1L1, SR-BI, ABCA1, ABCG5, CD36, and sterol-regulatory element binding protein (SREBP), a transcription factor that controls genes related to lipid and cholesterol metabolism, subcellular fractionation was carried out to examine NPC1L1 protein expression. Molecular strategies were also devised in the Caco-2 cell line, a reliable human intestinal model for lipid metabolism and lipoprotein assembly (16, 17), to determine the importance of NPC1L1 in intestinal cholesterol transport and metabolism. Finally, studies were performed to establish the relationship between NPC1L1, SR-BI, ABCA1, ABCG5, CD36, and sterol-regulatory element binding protein (SREBP), a transcription factor that controls genes related to lipid and cholesterol homeostasis (18).

MATERIALS AND METHODS

Human gut specimens

Adult human small intestine was obtained from three healthy organ donors (25–40 years) with the generous collaboration of Dr. C. Malo and Quebec-Transplant after consent forms were signed. The entire procedure was approved by the Ethics Committee of the Faculty of Medicine, Université de Montréal. The small intestine was divided into three segments: duodenum (first 25 cm), jejunum (140 cm), and ileum. The tissues were rinsed with ice-cold saline and frozen at −80°C. Additional intestine specimens were obtained from patients with Crohn’s disease (30–45 years) who had undergone surgical resection of different areas of the digestive tract. Normal tissue found adjacent to the resected pathological tissues was used in all cases, as ascertained by routine hematoxylin/eosin staining. Only tissues from the same locations were used, to avoid variability. Studies were approved by the Institutional Review Committee for the Use of Human Resected Material at the Centre Hospitalier Universitaire de Sherbrooke/Faculté de Médecine and by the Ethics Committee of Sainte-Justine Hospital.

Tissue preparation for electron microscopy

Intestinal specimens were fixed by immersion in 1% glutaraldehyde and 0.1 M phosphate-buffered saline (pH 7.4) for 2 h at 4°C and embedded in Lowicryl K4M at −20°C according to our previously described procedures (19). Tissue blocks were examined by light microscopy to select well-oriented villous tips. Thin sections (60–80 nm) of the different tissue blocks were mounted on nickel grids with carbon-coated Parlodion film and processed for immunocytochemistry.

Immunocytochemical labeling

Protein A-gold immunocytochemical techniques were used to detect the presence of NPC1L1 in intestinal tissue, as described previously (20–22). Briefly, the tissue sections were washed initially in distilled water, incubated for 5 min on a drop of PBS containing 1% ovalbumin, and transferred subsequently to a drop of the PBS-diluted antibody (see below). After incubation (90 min) at room temperature, the grids were rinsed with PBS to remove unbound antibodies. They were transferred to the PBS-ovalbumin (3 min) and incubated on a drop of protein A-gold (pH 7.2) for 30 min at room temperature. The tissue sections were then washed thoroughly with PBS, rinsed with distilled water, and dried. Sections were stained with uranyl acetate and lead citrate before examination with a Philips 410 electron microscope. Polyclonal antibodies against NPC1L1 were used at a dilution of 1:100 in combination with protein A-gold complexes, which were prepared using 5 or 10 nm gold particles according to our established techniques (20). Control experiments were performed to assess the specificity of the results. Preimmune rabbit serum (diluted 1:10) was used on tissue sections before incubation with the protein A-gold complex. Incubations were also performed with the protein A-gold complex alone, omitting the antibody step to test for nonspecific adsorption of the protein A-gold complex to tissue sections.

Cell culture

Caco-2 cells (American Type Culture Collection, Rockville, MD) were grown at 37°C with 5% CO2 in DMEM (Gibco-BRL, Grand Island, NY) containing 1% penicillin/streptomycin and 1% MEM nonessential amino acids (Gibco-BRL) and supplemented with 10% decomplemented FBS (Flow, McLean, VA). Caco-2 cells (passages 30–40) were maintained in 75 cm2 flasks (Corning Glass Works, Corning, NY). Cultures were split (1:6) when they reached 70–90% confluence, using 0.05% trypsin-0.5 mM EDTA (Gibco-BRL). For individual experiments, cells were plated at a density of 1 × 106 cells/well on 24.5 mm polycarbonate Transwell filter inserts with 0.4 μm pores (Costar, Cambridge, MA) in DMEM (as described above) supplemented with 5% FBS. The inserts were placed onto six-well culture plates and cultured for 20 days, at which time Caco-2 cells are highly differentiated and appropriate for lipid metabolism (23, 24).

Transfection of Caco-2 cells

Caco-2 cells (1 × 106 cells/well) were plated on six-well plates using media conditions as described above and incubated for 24 h. Cells were transfected with vector-based small interfering RNA (siRNA) against NPC1L1 (3 μg/well) or mock plasmid (3 μg/well) using Lipofectamine 2000 and serum-free medium according to the manufacturer’s recommendations (Invitrogen, Carlsbad, CA). All transfections were done in triplicate, and cells were harvested at 48 h after transfection. The optimal amount of Lipofectamine 2000 and cell number were determined before transfection using green fluorescent protein (GFP)-expressing plasmids.

Establishment of stable RNA interference Caco-2 cell lines

Caco-2 cells were transfected with vector-based siRNA as described above. Forty-eight hours after transfection, cells were transferred to plates and grown in medium containing 100 μg/ml hygromycin B (Invitrogen) until distinct clones could be isolated. Single clones were transferred to 96-well plates, grown until confluence, and transferred to flasks. Thereafter, cells were...
cultivated until 21 days after confluence and used only after the validation of NPC1L1 suppression.

Vector construction, transformation, and sequencing

pSilencer 2.1-U6 hygro (Ambion, Austin, TX) was linearized with both BamHI and HindIII and gel-purified to remove the digested GFP insert. For siRNA expression against NPC1L1, the two complementary oligonucleotides used were 5’-GATCCGCTTCCATGACCAGCATTCAGAGAAATGCTGGTCATGGAAGGCTTTTTTGGAAA-3’ and 5’-AGCTTTTCCAA-AAAAGCTTTCCATGACCAGCATTCAGAGAAATGCTGGTCATGGAAGGCGG-3’. They were annealed and cloned into the linearized pSilencer 2.1-U6 hygro vector. Escherichia coli competent cells were transformed with the resulting ligation product, plated on Luria-Bertani plates containing 50 µg/ml ampicillin, and grown overnight at 37°C. Clones were picked, and plasmid DNA was isolated and digested with BamHI and HindIII to confirm the presence of the 63 bp siRNA template. The insert was sequenced with a Beckman Coulter CEQ 2000 XL sequencer using the primers 5’-AGCGATTAAGTTGGGTA-3’ and 5’-GTAATACGACTGACTATAGGG-3’. We named the vector expressing siRNAs against NPC1L1 psiNPC1L1. Caco-2 cells transfected with pSilencer 2.1-U6 hygro plus GFP insert were used as mock controls.

RT-PCR

PCR experiments for NPC1L1, SR-BI, SREBP, CD36, ACAT, HMG-CoA reductase, ABCA1, and ABCG5 genes as well as β-actin (as a control gene) were performed using the GeneAmp PCR System 9700 (Applied Biosystems). Approximately 30–40 cycles of amplification were used at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. Amplicons were visualized on standard ethidium bromide-stained agarose gels. Under these experimental conditions relative to RT-PCR, 34–36 cycles corresponded to the linear portion of the exponential phase from human Caco-2 cells (Fig. 1).

Western blots

To assess the presence of NPC1L1, intestinal tissues or Caco-2 cells were homogenized and adequately prepared for Western blotting as described previously (25). Proteins were denatured in sample buffer containing SDS and β-mercaptoethanol, separated on a 4–20% gradient SDS-PAGE gel, and electroblotted onto nitrocellulose membranes. Nonspecific binding sites of the membranes were blocked using defatted milk proteins followed by the addition of rabbit polyclonal antibodies generated against the N-terminal region of NPC1L1, spanning amino acids 524–541. Moreover, no signal was noted addition of rabbit polyclonal antibodies generated against the N-terminal region of NPC1L1, spanning amino acids 524–541. In addition, no signal was noted with both primary antibody was detected with species-specific HMG-CoA reductase activity assay

Enzymatic activity was assayed as described previously (24, 26). The reaction mixture contained 100 mM potassium phosphate (pH 7.4), 200 µg of cellular protein, 20 mM glucose-6-phosphate, 12.5 mM dithiothreitol, 2.5 M NADP, and 1.2 units of glucose-6-phosphate dehydrogenase. Initiation of the reaction was done by the addition of [14C]HMG-CoA (200 Bq/nmol) for 30 min at 37°C. The [14C]mevalonate formed was converted into lactone by the addition of 10 N HCl, isolated by TLC, and counted using an internal standard to correct for incomplete recovery.

ACAT activity assay

The activity of ACAT was determined by adding 5 nmol of [14C]oleoyl-CoA (specific activity ~ 167 Bq/nmol) to the mixture containing 200 µg of cellular protein to initiate the reaction in a buffer solution (pH 7.5) consisting of cholesterol, 0.04 M KH2PO4, 50 mM NaF, 0.25 M sucrose, and 1 mM EDTA. After incubation for 10 min at 37°C, the reaction was stopped by adding chloroform-methanol (2:1, v/v) followed by free cholesterol (FC) and cholesteryl ester (CE) as carriers. The FC and CE formed were isolated by TLC and counted.

Isolation of the brush-border membrane

Mucosa from human jejunal tissue was scraped. The brush-border membrane from Caco-2 cells and jejunal mucosa was prepared as described previously (27). Briefly, homogenization was performed in 2 mM Tris-HCl and 50 mM mannitol (pH 7.1) containing 10 µg/ml aprotinin and leupeptin as protease inhibitors. The homogenate was clarified by centrifugation at 500 g for 10 min, and GaCl2 was added to a final concentration of 10 mM. After incubation at 15 min on ice, the preparation was centrifuged at 1,500 g for 10 min to pellet intracellular and
basolateral membranes. Finally, the supernatant was centrifuged at 48,000 g for 1 h to obtain a pellet of microvillar membranes.

Statistical analysis

Data from the experiments were analyzed using Student’s t-test. Reported values are expressed as means ± SEM. Statistical significance was accepted at $P < 0.05$.

RESULTS

Experiments were first conducted to study the distribution of NPC1L1 along the human small intestine and in Caco-2 cells. Samples with equal quantities of protein were electrophoresed by SDS-PAGE and then immunoblotted (Fig. 2A). Densitometric estimation of the NPC1L1 visualized on the immunoblot showed that the jejunum exhibited a significantly higher NPC1L1 protein content than the duodenum and ileum. Similarly, Caco-2 cells were endowed with a substantial NPC1L1 signal intensity. Overall, these data suggested a role for NPC1L1 in intestinal epithelial cells. Confirmation was obtained by RT-PCR analysis illustrating the predominance NPC1L1 mRNA in the jejunum region (Fig. 2B).

To establish the expression of NPC1L1, indirect immunofluorescence was carried out in the human small intestine. Figure 3A illustrates the presence and localization of NPC1L1 in columnar epithelial cells. Immunofluorescent staining was mostly visualized in the brush-border membrane. In an effort to better understand the cellular localization of NPC1L1, protein A-gold immunocytochemical techniques were applied on thin sections incubated with specific antibodies to disclose NPC1L1 in normal human intestinal biopsies. Electron microscopic immunocytochemical studies revealed significant immunogold labeling over the microvilli associated with the luminal plasma membrane and over the endosomal compartment...
and lysosomes (Fig. 3B). Labeling was also detected over the mitochondria and endoplasmic reticulum. Most of the labeling over the mitochondria was associated with the peripheral membranes; few particles were found inside the mitochondria matrices (Fig. 3B). Under the control conditions tested, labeling was markedly reduced or eliminated, demonstrating its specificity (data not shown). When the protein A-gold immunocytochemical technique was applied on Caco-2 cells, similar findings were noted.

To confirm these morphological data, we fractionated epithelial cells isolated from human jejunum (Fig. 4). Again, analyses of the subcellular fractions corroborated its expression in the aforementioned organelles (i.e., microsomes, lysosomes, and mitochondria). Furthermore, Western blotting of brush-border membranes purified from human jejunal mucosa once more disclosed the presence of NPC1L1 in microvilli (Fig. 4), which is compatible with the high-resolution immunogold findings. Additionally, analysis of gene expression disclosed the presence of NPC1L1 in Caco-2 cells and human jejunum along with SR-BI and brush-border markers (i.e., sucrase and aminopeptidase) (Fig. 5A). Subsequently, studies on isolated microvilli revealed the colocalization of NPC1L1 with SR-BI as well as with brush-border markers (i.e., sucrase-isomaltase and aminopeptidase with polyclonal antibodies raised in rabbits in our unit) (Fig. 5B). Altogether, our results suggest apical and intracellular locations of NPC1L1 in Caco-2 cells and human small intestine with appearance in the brush-border membrane, a crucial site for lipid absorption.

The next series of experiments aimed at examining whether NPC1L1 functions as a cholesterol transporter in human epithelial cells. To this end, we first evaluated the effects of RNA interference (RNAi)-mediated NPC1L1 knockout on Caco-2 cells. The RNAi experimental approach resulted in a significant reduction in NPC1L1 gene expression in Caco-2 cells (Fig. 6A). Native transcripts examined by RT-PCR were suppressed by 87% compared with the mock controls transfected with the pSilencer 2.1-V6 hygro vector (Fig. 6A). There was a corresponding 90% decrease in NPC1L1 protein expression evaluated by Western blot (Fig. 6B). We also confirmed the reduction in NPC1L1 protein expression with electron microscopy using protein A-gold, which revealed less gold particle content in transfected Caco-2 cells (results not shown). The molecular manipulation procedure did not significantly modify cell growth, differentiation, and viability, as listed in Table 1. Interestingly, NPC1L1 expression did not

Fig. 4. Representative distribution of NPC1L1 in subcellular fractions prepared from human jejunum. After tissue homogenization and subcellular fractionation by discontinuous sucrose density gradient ultracentrifugation, samples (10 μg of protein) were subjected to SDS-PAGE and Western blotting. The purity of the brush-border membrane was assessed by the enrichment of sucrase and aminopeptidase-N. The activities of glucose-6-phosphatase (a marker of microsomes), galactosyltransferase (a marker of Golgi organelles), acid phosphatase (a marker of lysosomes), and succinate dehydrogenase (a marker of mitochondria) were not detected in isolated apical membranes. Mv, microvilli; Mic, microsomes; Ly, lysosomes; Mit, mitochondria.

Fig. 5. Gene and protein expression of NPC1L1 in Caco-2 cells and human jejunum. A: PCR analysis was performed to analyze mRNA of NPC1L1 and SR-BI as well as markers of the brush-border membrane (i.e., sucrase and aminopeptidase). B: Subsequent assays with Western blot were carried out to examine the colocalization of NPC1L1 with brush-border proteins. To determine the purity of the subcellular fractions, the activities of marker enzymes were measured. The enrichment of the various enzyme activities was as follows: 11.2 for sucrase activity in brush-border membranes; 4.2 for glucose-6-phosphatase in microsomes; 3.4 for acid phosphatase in lysosomes; and 3.5 for succinate dehydrogenase in mitochondria.
markedly differ between undifferentiated, preconfluent cells and differentiated, postconfluent Caco-2 cells (Fig. 7).

Cholesterol uptake was then determined in control mock cells and genetically modified intestinal cells. NPC1L1 gene knockdown caused markedly decreased ability of Caco-2 cells to capture micellar [14C]FC and to convert it to its esterified form, CE (Fig. 8). Moreover, the levels of CE were also decreased in the culture medium. Thus, these experiments emphasize the important role of NPC1L1 in intestinal cholesterol transport and metabolism.

Next, we determined the impact of NPC1L1 absence on the regulatory sterol enzymes: HMG-CoA reductase (EC 1.1.1.34), the rate-limiting step in cholesterol synthesis, and ACAT (EC 2.3.1.26), an integral protein present in the rough endoplasmic reticulum that catalyzes the formation of CE from FC and fatty acyl-CoA. NPC1L1 gene knockdown in Caco-2 cells led to an increase in the activity (Fig. 9A) and mRNA (Fig. 9B) of HMG-CoA reductase. On the other hand, the activity (Fig. 10A) and mRNA (Fig. 10B) of ACAT were decreased in cells deficient of the NPC1L1 gene.

After these observations, we determined the gene expression of SREBP, a critical transcription factor governing lipid homeostasis. The suppression of NPC1L1 in Caco-2 cells upregulated SREBP mRNA (Fig. 11). Other proteins have been located mainly to the brush-border membrane of the epithelial cells and have been shown to be involved in the facilitated uptake of dietary cholesterol (8–11, 13, 28). Such information prompted us to examine whether the targeted disruption of NPC1L1 alters the profile of potential cholesterol transporters: SR-BI, ABCA1, NPC1L1, Niemann-Pick C1-Like 1. To evaluate the possibility of cell-damaging effects by the transfection of interfering RNA against NPC1L1, cell monolayer integrity was investigated by the measurement of lactate dehydrogenase, protein/DNA ratio, and trypan blue exclusion. Cell differentiation was assessed by the determination of lactase, lactate dehydrogenase, and alkaline phosphatase. Values are means ± SEM for three separate experiments.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>NPC1L1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrase (µmol/min/g protein)</td>
<td>41.5 ± 3.7</td>
<td>39.3 ± 4.4</td>
</tr>
<tr>
<td>Lactase (µmol/min/g protein)</td>
<td>3.4 ± 0.5</td>
<td>3.7 ± 0.7</td>
</tr>
<tr>
<td>Alkaline phosphatase (µmol/min/g protein)</td>
<td>30.9 ± 3.1</td>
<td>28.8 ± 2.7</td>
</tr>
<tr>
<td>Lactate dehydrogenase (µmol/min/g protein)</td>
<td>7.7 ± 1.2</td>
<td>8.5 ± 1.8</td>
</tr>
<tr>
<td>Cell protein/DNA ratio</td>
<td>&gt;92%</td>
<td>&gt;90%</td>
</tr>
<tr>
<td>Trypan blue exclusion (%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NPC1L1 and intestinal cholesterol transport
ABCG5, and CD36. Disruption of the NPC1L1 gene in Caco-2 cells diminished SR-BI mRNA and resulted in unchanged ABCA1, ABCG5, and CD36 mRNA levels (Fig. 11).

**DISCUSSION**

Despite significant advances, the molecular mechanisms of dietary cholesterol uptake from the intestinal lumen remain poorly defined. Not long ago, Altmann et al. (14) suggested that NPC1L1, a recently identified relative of NPC1, is crucial for intestinal cholesterol absorption. Most of their important experiments were carried out in NPC1L1-null mice. To gain a further understanding of the localization and function of NPC1L1 in human intestinal tissue, we performed immunocytochemical, cellular fractionation, and molecular studies particularly in Caco-2 epithelial cells. Our data revealed its predominance in the jejunum along the small intestine and the NPC1L1 enrichment content in Caco-2 cells compared with different regions of human intestine. We were able to detect NPC1L1 at the brush-border membrane with the use of cell fractionation and high-resolution immunoelectron microscopy. NPC1L1 also resided in subcellular compartments of the human enterocyte, including the lysosomes and mitochondria. These findings prompted us to determine whether NPC1L1 functions as a cholesterol transporter in human enterocytes. Clearly, inactivation of NPC1L1 led to defects of cholesterol transport, variations in key regulatory sterol enzymes (HMG-CoA reductase gene expression and activity).
CoA reductase and ACAT), and high gene expression of SREBP, suggesting the role of NPC1L1 in cholesterol homeostasis. Subsequent efforts documented that NPC1L1 knockdown specifically reduced the transcripts of SR-BI, a protein present in the apical membrane, which is suggested to play a role in cholesterol uptake.

Knowledge of the location of NPC1L1 is extremely important because it provides clues to the potential function of this transporter. NPC1L1 resided with sucrase-isomaltase, aminopeptidase, and SR-BI, characteristic proteins of the brush-border membrane. Using the high-resolution immunogold technique, labeling was mainly over microvilli and intracellular organelles of the enterocyte and confirmation was obtained by cell subfractionation. If NPC1L1 is confined mainly to the apical microvillous membrane, what is its role in intestinal epithelial cells? Is it involved in cholesterol absorption in humans? Does it influence cholesterol metabolism? To tackle these specific questions, we generated Caco-2 knockdown cells of NPC1L1, which appear to be phenotypically normal and do not exhibit abnormalities in cell growth, differentiation, and viability. Interestingly, RNAi-mediated NPC1L1 disruption in human Caco-2 cells significantly decreased cholesterol uptake, a transport phenomenon indistinguishable from that recently described for NPC1L1-deficient mice (14).

Subsequently, it was necessary to determine whether NPC1L1 suppression affects other cholesterol transporters positioned on the brush-border membrane. Indeed, several lines of evidence support the hypothesis that protein-facilitated mechanisms are involved in cholesterol uptake by the enterocyte (for reviews, see 29–31). SR-BI and CD36 have been proposed by various laboratories to mediate intestinal cholesterol absorption (8–11, 28). Whereas no impairment was noted in ABCA1, ABCG5, and CD36 transcripts, targeted disruption of NPC1L1 appears to exert a downregulation of SR-BI without altering CD36 gene expression. Apparently, deficiency in NPC1L1 interferes with potential molecular targets for cholesterol absorption. The regulatory connection between NPC1L1 on the one hand and SR-BI on the other is not characterized. Preliminary attempts to reconstitute cholesterol transport activity in cells by overexpression of NPC1L1 have been unsuccessful, indicating that additional proteins are necessary to reconstitute an active cholesterol transporter (14). Further studies are needed to clarify the mechanistic link between NPC1L1 and other potential cholesterol carriers.

After uptake, the FC is reesterified in the enterocytes by the action of ACAT, an integral enzyme present in the rough endoplasmic reticulum that catalyzes the formation of CE from cholesterol and fatty acyl-CoA. The resulting CE is packaged into chylomicrons and secreted from the basolateral membrane of the enterocyte into the lamina propria, from which it enters the lymph. The regulatory mechanisms of ACAT activity are not fully understood. Cholesterol availability may be one of the regulatory mechanisms of ACAT activity (32, 33). Feeding cholesterol to animals significantly increased the activity of intestinal ACAT (34–36). The increase in activity is thought to result from an expansion of the finite cholesterol substrate pool for ACAT. If cholesterol mediated by NPC1L1 is targeted to this pool, it is conceivable that NPC1L1 knockdown would lead to reduced ACAT activity. Based on the data in Fig. 10, this assumption turned out to be true. Deficiency of NPC1L1 strongly affected ACAT activity and mRNA. Conversely, intracellular low cholesterol levels enhanced HMG-CoA reductase activity and mRNA, which indicated that CaCo-2 cells sensed cholesterol depletion caused by NPC1L1 knockdown.

Analysis of the NPC1L1 putative promoter region identified the presence of SREBP (37–40), which is implicated in the sterol regulation of gene expression (41, 42). SREBPs are membrane-bound transcription factors that activate the synthesis and uptake of cholesterol and fatty acids (42). We have found that SREBP mRNA was increased in Caco-2 cells deprived of NPC1L1. High SREBP levels may explain the positive regulation of HMG-CoA reductase.

NPC1L1 immunostaining was associated with the apical membrane of enterocytes, with regions displaying punctuate appearance of the NPC1L1 distribution. The NPC1L1 immunostaining expanded to submicrovillar organelles and structures resembling endosomes, as illustrated by immunogold microscopy. Intracellular organelles have also been found in HepG2 cells (43). Our findings are consistent with a model in which NPC1L1 may be translated from intracellular organelles or endosomes to the plasma microvillous membrane in the enterocyte. Anchoring mechanisms may allow the retention and functioning of NPC1L1. Accordingly, growing evidence in cultured HepG2 cells suggests that NPC1L1 protein traffic through the plasma membrane and intracellular compartments through the endocytic recycling pathway, probably regulated by cellular cholesterol availability (44). Interestingly, Yu et al. (44) have shown that acute cholesterol depletion relocates NPC1L1 to the cell surface, preferentially to a newly formed apex-like subdomain, resulting in an increased uptake of FC through NPC1L1, which can be dose-dependently inhibited by a novel cholesterol absorption inhibitor, ezetimibe. Additional studies are obviously necessary to test this concept in intestinal tissues.

In conclusion, significant suppression of NPC1L1 in Caco-2 cells impaired cholesterol uptake, modified key regulatory sterol enzymes, and induced SREBP gene expression. These findings suggest the important role of NPC1L1 in intestinal cholesterol homeostasis. However, additional work is required to highlight the cross-talk mechanisms between NPC1L1 and SR-BI.

This work was supported by grants from the Canadian Institutes of Health Research and Valorisation Recherche Québec (Grant VRQ-2201-146). The authors thank Mrs. Schohraya Spahis for her expert assistance.

REFERENCES


